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Aspergillus nidulans verA Is Required for Production of the Mycotoxin Sterigmatocystin

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Aspergillus nidulans **produces the carcinogenic mycotoxin sterigmatocystin (ST), the next-to-last precursor in the aflatoxin (AF) biosynthetic pathway found in the closely related fungi *Aspergillus flavus* and *Aspergillus parasiticus*. We identified and characterized an *A. nidulans* gene, *verA*, that is required for converting the AF precursor versicolorin A to ST. *verA* is closely related to several polyketide biosynthetic genes involved in polyketide production in *Streptomyces* spp. and exhibits extended sequence similarity to *A. parasiticus ver-1*, a gene proposed to encode an enzyme involved in converting versicolorin A to ST. By performing a sequence analysis of the region 3′ to *verA*, we identified two additional open reading frames, designated ORF1 and ORF2. ORF2 is closely related to a number of cytochrome P-450 monooxygenases, while ORF1 shares identity with the gamma subunit of translation elongation factor 1. Given that several steps in the ST-AF pathway may require monooxygenase activity and that AF biosynthetic genes are clustered in *A. flavus* and *A. parasiticus*, we suggest that *verA* may be part of a cluster of genes required for ST biosynthesis. We disrupted the *verA* coding region by inserting the *A. nidulans argB* gene into the center of the coding region and transformed an *A. nidulans argB2* mutant to arginine prototrophy. Seven transformants that produced DNA patterns indicative of a *verA* disruption event were grown under ST-inducing conditions, and all of the transformants produced versicolorin A but negligible amounts of ST (200-fold to almost 1,000-fold less than the wild type), confirming the hypothesis that *verA* encodes an enzyme necessary for converting versicolorin A to ST.**

Toxic fungal metabolites, which are called mycotoxins, are produced by numerous fungi, including fungi commonly found as contaminants of food or feed products and fungi used for industrial production of food additives and pharmaceuticals. Many mycotoxins are classified as polyketides, which are bioreactive secondary metabolites that are synthesized like fatty acids (20). Although some polyketides (e.g., antibiotics and cancer therapeutic drugs) are beneficial, the mycotoxigenic polyketides present serious health and economic problems. Of particular concern are the carcinogenic polyketides aflatoxin (AF) and sterigmatocystin (ST), which are produced by many species of the genus *Aspergillus*. It has been proposed that both of these compounds are end products of the same lengthy biosynthetic pathway: initial polyketide precursor→norsolorinic acid→averantin→averufanin→averufin→versiconal hemiacetal acetate→versicolorin B→versicolorin A→demethylsterigmatocystin→ST→O-methylsterigmatocystin→AF B_1 (5, 41). AF and ST cause mammalian liver cancers (1a, 16) and are found in foods, including peanuts, corn, tree nuts, cheese, milk, and meat (8, 14, 21, 28, 32).

In recent studies workers have identified several of the enzymes and genes required for AF biosynthesis in *Aspergillus flavus* and *Aspergillus parasiticus* (10, 11, 23, 29, 34, 41, 43). However, little effort has been made to investigate ST biosynthesis in ST-producing *Aspergillus* spp. As many as 20 *Aspergillus* species, including *Aspergillus nidulans* (2, 17), a filamentous fungus widely used to study basic biological problems (39), have been reported to produce ST (12). Although current federal guidelines on AF contamination do not cover ST, the possible effects of ST on human health cannot be ignored. It has been shown that many of ST's bioreactive activities parallel those of AF, although slightly higher concentrations of ST are usually needed to produce the same effect (3, 33). Because ST can be produced in greater quantities than AF in certain foods (19) and may be found in a larger variety of foods and environments, it is important to understand parameters that control ST biosynthesis (15, 24, 28, 32, 37). In this paper we describe the isolation and characterization of an *A. nidulans* gene required for ST production, *verA*. Disruption of the *verA* coding region results in an inability to convert versicolorin A to ST.

MATERIALS AND METHODS

Fungal strains and growth conditions. *A. nidulans* FGSC 26 (*biA1 veA1*) and rM31 (*pabaA1 biA1 argB2 veA1*) were maintained as silica stocks and were grown on minimal medium for the production of conidia. Strain rM31 was isolated as the meiotic progeny of strains PW1 (*biA1 argB2 methG1 veA1*) and FGSC 237 (*pabaA1 yA2 trpC801 veA1*). To induce ST biosynthesis, the fungal isolates were grown either on oatmeal porridge or in YEC (0.2% yeast extract, 5.0% corn steep liquor).

Nucleic acid manipulations. An *A. nidulans* genomic cosmid library (1, 7) was screened with an internal fragment from the *A. parasiticus ver-1* gene, pBSV2, which has been predicted to encode a keto-reductase required for conversion of versicolorin A to ST (34). All hybridization experiments with ^{32}P-labeled *ver-1* were carried out at 68°C, and the membranes were washed twice in 2× SSPE–0.1% sodium dodecyl sulfate (SDS) (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) at room temperature.

The chromosome containing the *A. nidulans ver-1* homolog was identified by probing a nylon membrane containing the separated chromosomes from *A. nidulans* FGSC 4 (kindly provided by Xiaoling Xuei and Paul Skatrud, Eli Lilly Corp.,

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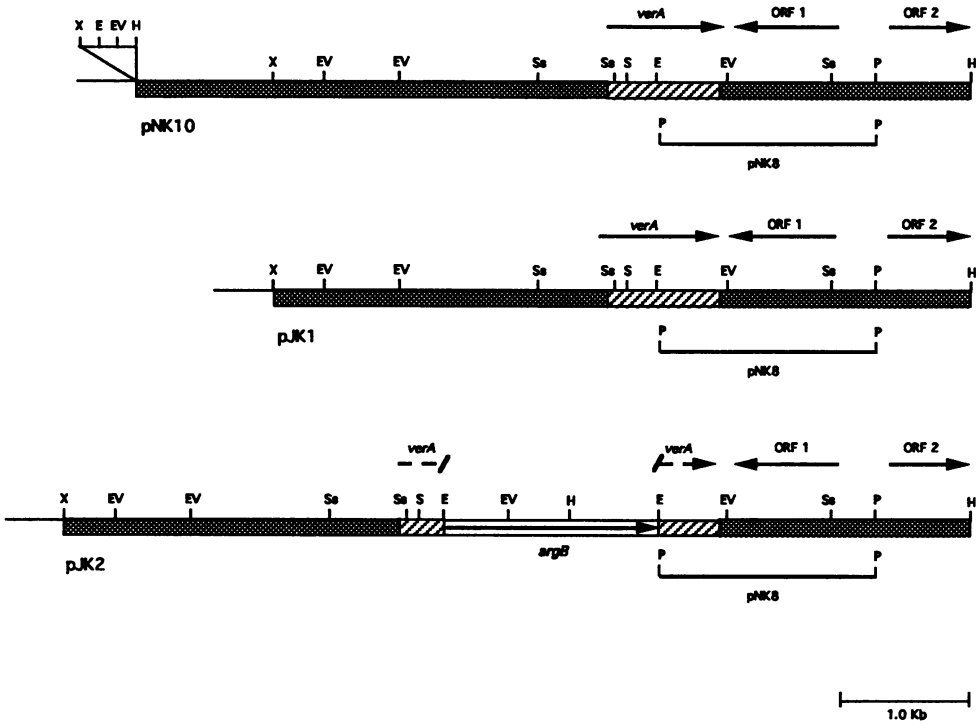

FIG. 1. Restriction map of the *verA* genomic region. pNK10 contains the entire *verA* gene within a 6.6-kb *Hin*dIII fragment. pJK1 was constructed from pNK10 by removing a 1.1-kb fragment that contained polylinker sequences including an *Eco*RI site. A 1.8-kb *Eco*RI fragment containing *argB*$^+$ was inserted into the *verA* coding region to give pJK2. pNK8 was used as a DNA probe in genomic blots to demonstrate disruption of *verA* and contains 509 bp of *verA* in a 1.8-kb *Pst*I fragment. *verA* is indicated by the diagonal lines, and the arrows indicate the directions of transcription for *verA*, *argB*, ORF1, and ORF2 (described in the text). Abbreviations: E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Ss, *Sst*II; X, *Xba*I.

Indianapolis, Ind.) with radioactively labeled pNK10 DNA, using standard methods (31). After prehybridization for 3 h in a solution containing 5× SSC, 50 mM $NaPO_4$ (pH 7.2), 0.4% SDS, 5× Denhardt's solution, 2.5 mM EDTA, 0.5% dextran sulfate, and 0.1 mg of salmon sperm DNA per ml (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the denatured probe was added, and hybridization was carried out for 24 h at 60°C. The membrane was washed at 60°C for 15 min in 2× SSC and then for 15 min in 1× SSC and then subjected to autoradiography.

The *verA* disruption plasmid, pJK2, was constructed in two steps (Fig. 1). First, pJK1 was constructed by digesting pNK10 with *Xba*I and religating. This removed a 1.1-kb fragment that contained a polylinker sequence including an *Eco*RI site. Next, pJK1 was digested with *Eco*RI and religated in the presence of a 1.8-kb *Eco*RI fragment containing *argB*$^+$ (obtained from pSalArgB) to give pJK2. pJK2 was linearized by double digestion with *Xba*I and *Pst*I, and the vector sequence was removed prior to transformation. Transformation, extraction of transformant genomic DNA, restriction enzyme digestion, gel electrophoresis, Southern blotting and hybridization were performed by using previously described protocols (31). All transformants were isolated from single spores prior to analysis.

Analysis of ST production. Beakers containing 3 g of oatmeal porridge were inoculated with 100 μl of a suspension containing 1×10^6 spores per ml. After 6 days of growth at 30°C, the cultures were killed by adding 15 ml of acetone. The beakers were gently agitated for 30 min, and then 15 ml of chloroform was added to each beaker. After an additional 30 min of agitation, each solution was passed through anhydrous sodium sulfate and collected in a beaker. After evaporation, each extract was resuspended in 1.5 ml of chloroform, and a 10-μl portion of each extract was spotted onto a thin-layer chromatography (TLC) plate (Analtech). An ST standard (Sigma) and a versicolorin A standard (a semipure standard previously prepared from extracts of *A. parasiticus* SRRC 164) were also spotted onto the TLC plates. The plates were developed with either benzene-glacial acetic acid (95:5, vol/vol) or toluene-ethyl acetate-glacial acetic acid (80:10:10, vol/vol/vol). After the plates were developed, they were sprayed with aluminum chloride, which enhanced the visualization of ST under long-wavelength (365-nm) UV light (38). A nonreplicated high-performance liquid chromatography (HPLC) analysis of the ST in culture extracts was performed by F. Neeley, Eli Lilly Corp., Indianapolis, Ind.

Nucleotide sequence accession number. The GenBank accession number for *A. nidulans verA* and flanking sequences encoding open reading frames (ORFs) ORF1 and ORF2 is L27825.

RESULTS

Isolation of *verA* and determination of the genomic sequence. The *A. nidulans verA* gene was isolated by using a fragment of the *A. parasiticus ver-1* gene as a heterologous probe to screen an *A. nidulans* cosmid genomic library, and one positive clone, pL24B3, was identified. The *ver-1* hybridizing region was localized to a 6.6-kb *Hin*dIII fragment and was subcloned into pBluescript SK$^-$ to give pNK10 (Fig. 1).

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   1 ATGCCCCAGAAGGGAGCACCGCTGTAGGCGCAAGCTGACCGAGTTGGGAGCGCAAAGCGTGGCCGCGGCTGCGATTGCGCTCGCATACCTGCTTCACAAG  100

 101 TCGCCGTACGTTTTCCCGGTGATCGGGTGCCGGACGGTCGAGCAGCTGGAGGCGAATATACGCCTCGGTGTAGAGCTCAGTGATGAGGAAATGTACGAGA  200

 201 TTGAAGACACGATCCCTTTTGATGTCGGCTTCCCCATGGCGTTCTTATTCGAATCGCCCCAGCAGAAGTACCGTAGTGATATGACGACCAGGCATATCTG  300

 301 GCAGGTTACCTGCAATGCCCGGATCGAGAGTGTGCCTAAGCCGAGAGTATGTATCTCTCAACCTGAATTTATGATTTCGCTAATCGAACTTACCAGCCTA  400

 401 TCGAGCCAAAGCAGGGTACAAGCAGATGGATCGGAAGTAGTTCTCGGTAGCATTAGCCAAGCATCGGGTCCCGAGCGTTCAAGTATTTTATATATGAGCC  500

                                                                - - - - H - - - - - - - - -
                                                        verA-> M S S S D N Y R L D G K V A L V
 501 TTGTTTCCTTCCTATGTCATGGTAGCCAGTATCCATAAGGTATAGGAATCAACCATGTCCTCCTCCGATAATTACCGTCTCGATGGAAAAGTCGCTCTGG  600

      - - - - - - - - - - - - - - - - E R - - - - - - - - - - H E - R P R
      T G A G R G I G A A I A V A L G Q P G A K V V V N Y A N S R E A A
 601 TAACTGGGGCTGGCCGCGGCATCGGAGCAGCCATCGCCGTAGCCCTCGGTCAGCCGGGCGCGAAGGTCGTCGTCAACTACGCTAACTCCCGTGAGGCCGC  700

     R - W L N R S R R M V P M L S Q S - - - - - - - E - T A - - - A E
     E K V V D E I K S N A Q S A I S I Q A D V G D P D A V T K L M D Q
 701 AGAAAAGGTCGTCGACGAAATCAAGTCGAACGCTCAGAGCGCCATTTCCATTCAAGCCGATGTCGGTGACCCTGATGCCGTCACCAAACTGATGGATCAG  800

     T - R - - - - - - - - - - - - - - - - - - - - - L - - - - - E
     A V E H F G Y L D I V S S N A G I V S F G H V K D V T P D
 801 GCCGTTGAGCACTTCGGATACCTGGATATAGTCTCATCTAACGCGGGAATTGTCTCGTTCGGGCATGTCAAGGACGTTACGCCAGATGTATGCGTCCCAT  900

                                    - - - - - - - - - - - - - - - - - - -
                                    E F D R V F R V N T R G Q F F V A R E A Y
 901 CTCCTTACGAAAGTCCTGTAGAGCTCTGACCTCAGCAGGAATTCGACCGAGTATTTCGGGTCAACACGCGCGGACAGTTTTTCGTCGCCCGCGAGGCGTA  1000

     - - M - - - - - - - - - - - - - - C - - - - - K - - - - - - - - -
     R H L R E G G R I I L T S S N T A S V K G V P R H A V Y S G S K G
1001 TCGCCATCTGCGTGAAGGCGGACGCATCATCCTCACAAGTTCCAACACAGCCAGCGTCAAAGGCGTCCCCAGGCACGCTGTGTACTCGGGCTCTAAGGGG  1100

     - - - - - - - - M - - - - - - - - - - - - - - - - - - - - - - - -
     A I D T F V R C L A I D C G D K K I T V N A V A P G A I K T D M F L
1101 GCGATTGACACCTTTGTGCGGTGCCTAGCTATCGACTGCGGCGACAAGAAGATCACGGTCAACGCGGTCGCTCCCGGCGCCATCAAGACCGATATGTTTC  1200

      A - - - - - - - - - - - - - - - - - - -
      S V S R E Y I P N G E T F T D E Q V D E
1201 TATCCGTGTCGCGAGAGTATATCCCCAATGGGGAGACTTTTACTGATGAGCAGGTGGATGAGGTACGTTTGTCTTTGTGTCTAGTATCTACGGCGGCTGC  1300

              - - - - - - - - - - - - - - - - - - - - - - - - - - T - -
              C A A W L S P L N R V G L P V D V A R V V S F L A S D A A E
1301 TAACTGGACAGTGTGCCGCGTGGCTGTCGCCGCTAAATCGGGTCGGATTACCGGTTGACGTGGCCCGGGTGGTCAGCTTTCTAGCTTCAGATGCGGCCGA  1400

     - V - - - - - - - - - - - - - -
     W I S G K I I G V D G G A F R *
1401 ATGGATCAGTGGAAAGATTATTGGCGTTGATGGGGGGGCCTTTAGATAAGTCACATCATATACTTGAACTATATAGGGTAGACATGCAATGTTCGCTCCC  1500

1501 CGCTCGCTTACCGATATCTGCCGATCATCGTCAGCAACCATTAGGTCACGAAAAAAAGAGTATACTAAGAGTAAACATCCGTGCATGGTATGAACTTAGT  1600

1601 TGGGTACACCGCAGTTAGTCACACCGTACTTAAGTACACTCAGCGATTCACTTAGGCGGCTGAATCGGCATTTCATACTCTGCCAGCACCGGAGGCCCAG  1700
                                                       * K P P Q I P M E Y E A L V P P G

1701 CAACATCAACAACAATAGGCAAAGCATGCACACGCTCAAACCAGGTAGATAGGTTGCGGTGCTCATCCCTCCAGCGTTTATCAAGGAAAAACCGGAATGC  1800
     A V D V V I P L A H V R E F W T S L N R H E D R W R K D L F F R F A

1801 GCCCTGCACAATCCCGAGCACAAACAGATCAGCTAGGCTGAGGGTTTCCCCGACCAAGTACTCTCGCCCACAAAGATGGTTGTCAAGAATCTTTAGCCGT  1900
      G Q V I G L V F L D A L S L T E G V L Y E R G C L H N D L I K L R

1901 GCTAAAGTGTCATCTTTGCTTTGATATATGTTGTCAGCATTGAAGTTGGCTCGTCCGATGAGCGGGTTGAACCAGCCCCCTAACGCTGGGAGGATTTCGG  2000
      A L T D D K S Q Y I N D A N F N A R G I L P N F W G G L A P L I E

2001 TGATCCCGAAGGCCATCCAGCGAATGATGGAGGCATATTCTTGTCCGGTAGTCCCAAGTAAAGTCGTATTTGAATCTTGAGATGTTACTATACCTCTTAG  2100
     T I G F A M W R I I S A Y E Q G T T G L L T T N S D

2101 TCAGGAATTGAATAGATGGAATTGCAGTAGCAGCATGGTACCATAGAGAGCAATAGCAATAGATTCCGTCAATACGTACGCGTCGGCCCCCACAAACGTA  2200
                                                    Y L A I A I S E T L V Y A D A G V F T

2201 GGAATCTTGCCTAGAGGGTTGAGCTGGAGATACTCTTCGGTAGCATCTTTGAATGAAGTGATGGTCTTGATTTTCAGAGGCAAATTGTTCGCTTTTGCAA  2300
      P I K G L P N L Q L Y E E T A D K F S T I T K I K L P L N N A K A

2301 TCGCAAGAATCGCCAGCGACCGCGGGTTGAACGGGCGAGTGTACAGAGTGCCGAACGGCATTGCAGAAATATTCTCAATTCAGAGCTGATTCTCGTATTG  2400
     I A L I A L S R P N F P R T Y L T G F P M <-ORF1
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2401  TATGCTTGTGGCAACCTGCTAAATACAAATACTGACAGCAAATCAACTATATGTCAAGACCATGCCCTTCAGCTGTCCGCGTAACCCTAACTTCCCCCAG   2500

2501  GACAACGGCCTTCATCTTTCCCCGATCCGTGAAACGGTCCTCGTCCGCCATAACTTCGGGGCTGCTCATGACGGGGACAAACTCCTCGAAGGTGGCTTGG   2600

2601  CTCGCAAATTGGACAACAGCAAATGCGTCGAAGGTCAAATCGATCGAGTCGCCGGCCAGCGGGGTGACCGGTTGCTGCAGGTAGTGTCGGGTGTGGCTGA   2700

2701  CTGGAAAGGCCCTCCCGCCGAGTCGTTGCAGCAGGGGGATATGTTCGGTCTCCCAGTGGTTACGAAATTCGCTGGGTGTGAGGTCGCCGCGACGGGCTAC   2800

2801  AAGAATCAAGACAGTGAACATGGTGGAGTGAAAGTGCTGTGTATGTTTGTCCACACTTGCTTCCAGAATCTCGCGCAATACGCCTCTATATATGGCCTGT   2900

2901  CCCTATCTCGGTCGCCGAACGAACTAAACAATTATTCAGAGAGACTCTTCTTACATTTTTGTCATTGTTGCCAAAGTCACTTCACTCATTGCTGTCCTCC   3000

3001  AACCATGTACACAACTATCATCACAGCGGTATGCGTGCTATTCGCTCTTCACCTCCTGGACAGCTTCTATCAAGCGCGGCAGGAGGTATGGGCCCTCCAG   3100

                                                    ORF2->  M  P  S  F  S  L  L  T  G  H
3101  CGGGCAAACCTAGTACGAGCCCTCTGACCCAATGATTGGCTAGAGGACGATTAACTGGTGATACAAGCCCATGCCTTCTTTCAGCCTGCTGACCGGCCAC   3200

      F  G  A  L  K  Q  T  I  D  G  M  P  P  N  A  T  L  H  S  I  M  L  K  L  S  Q  K  F  R  S  G  M  F  Y
3201  TTTGGTGCCCTCAAACAAACCATCGATGGCATGCCGCCCAACGCAACCCTGCATAGCATTATGCTGAAATTGTCGCAAAAGTTCCGCTCAGGGATGTTCT   3300

        I  N  M  W  P  F  S  G  T  W  L  V  V  A  T  P  S  G  A  A  Q  I  Q  S  L  N  L  S  K  P  P  L  V
3301  ACATCAACATGTGGCCATTCAGCGGTACATGGCTCGTGGTCGCAACACCGTCTGGCGCGGCCCAGATCCAGAGTCTGAATCTTTCGAAGCCGCCGCTGGT   3400

       R  R  P  L  E  T  I  T  G  G  P  S  L  M  S  M  H  G  E  T  W  K  R  W  R  A  L  F  N  P  G  F  N
3401  GCGAAGACCGCTGGAGACTATCACCGGGGGCCCAAGCTTGATGAGTATGCATGGTGAAACATGGAAACGGTGGAGGGCACTGTTTAATCCAGGCTTTAAC   3500

      P  N  Y  L  I  G  L  A  P  L  I  A  D  E  V  V  V  F  C  E  Q  L  R  Q  K  A  R  T  G  T  V  F  Q  L
3501  CCCAACTACTTGATTGGGCTGGCGCCGCTGATCGCCGATGAGGTCGTTGTTTTTTGCGAGCAGCTACGGCAGAAGGCCAGAACAGGAACAGTTTTCCAGC   3600

        E  P  L  T  L  R  L  T  V  D  T  I  C  S  V  T  L  Y  V  V  T  P  V  G  R  W  P  F  L  T  P  D  L
3601  TTGAACCGCTCACTCTGAGGTTGACAGTTGATACGATTTGCTCTGTGACGTTGTATGTGGTTACTCCCGTTGGGCGATGGCCCTTTCTAACCCCTGACTT   3700

       E  I  H  S  S  T  T  K  L  R  T  T  P  L  P  Q  R  C  N  G  R  S  N  G  P  R  L  E  L  P  S  T  P
3701  AGAGATTCACAGCTCCACCACCAAACTCAGGACCACCCCCTTGCCTCAGCGCTGCAACGGCAGATCGAATGGGCCTCGTTTGGAACTACCTTCAACCCCT   3800

      L  R  R  Y  L  T  V  R  P  L  V  M  W  Y  N  N  R  L  M  N  R  F  I  D  Q  E  V  D  R  A  Y  R  E  Q
3801  TTAAGGCGGTACCTGACCGTGCGGCCTCTGGTGATGTGGTACAATAACCGCCTTATGAACCGCTTCATCGACCAAGAGGTTGACCGAGCGTACCGGGAGC   3900

        S  G  R  Q  S  K  S  V  I  S  L  A  L  R  D  Y  M  K  E  K  D  G  S  L  E  D  F  K  R  R  V  A  P
3901  AGTCTGGCCGTCAGTCGAAATCCGTGATCTCCCTCGCCCTCAGAGATTACATGAAAGAGAAAGATGGAAGTCTGGAAGACTTCAAACGACGTGTTGCGCC   4000

       Q  L  R  V  F  L  F  A  G  R  D  T  T  S  S  T  L  L  Y  A  F  Y  L  L  S  R  H  P  E  A  L  A  K
4001  ACAGTTACGGGTCTTTCTCTTCGCAGGTAGAGATACAACGAGCAGTACACTGCTCTATGCATTCTACCTGCTTTCCCGACATCCAGAGGCCCTAGCTAAG   4100

      V  R  L  E  H  D  Q  V  F  G  P  Y  H  Q  Q  V  H  E  K  I  H  Q  D  A  K  L  L  N  Q  L  P  Y  T  T
4101  GTGCGCTTAGAGCACGACCAGGTCTTCGGCCCATATCATCAACAAGTACACGAGAAAATCCACCAAGATGCGAAACTCCTCAACCAACTCCCCTACACAA   4200

        A  V  L  K  E  T  L  R  L  F  P  P  S  A  S  M  R  E  A  D  P  A  L  K  S  P  T  T  T  A  K  Y  I
4201  CAGCTGTCCTTAAAGAGACTCTGAGGCTCTTCCCTCCGTCTGCCTCCATGCGTGAAGCGGACCCGGCGTTGAAATCACCGACGACAACGGCCAAGTATAT   4300

       P  L  Q
4301  CCCACTGCAG  4310
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FIG. 2. Nucleotide sequence of a 4,310-bp region containing *verA*, ORF1, and ORF2. The putative translation start site of *verA* begins at nucleotide 555, and the termination codon starts at nucleotide 1446. A comparison of the amino acid sequences of the products of *A. nidulans verA* (bottom line) and *A. parasiticus ver-1* (top line) (34) is shown above the nucleotide sequence; dashes indicate that the amino acids in the two peptides were identical. The comparison shows the high degree of conservation between the two gene products. A putative NADPH binding site, GXGXXA, is underlined (starting at residue 20 for *verA* and residue 18 for *ver-1*). The intron positions in *verA* were predicted on the basis of sequence similarity to a *ver-1* cDNA. Two additional ORFs found downstream of *verA*, one similar to the gamma subunit of elongation factor 1 (ORF1) and the other related to several cytochrome P-450 monooxygenases (ORF2), are also shown.

Further analysis revealed that the similarity spanned the central *Eco*RI site, and by performing sequence analysis of the region we identified the *verA* gene and the coding region (Fig. 2). In addition, a sequence analysis of several thousand base pairs downstream from *verA* revealed two additional potential ORFs, designated ORF1 and ORF2 (Fig. 1). ORF1 and *verA* read in opposite orientations, and ORF1 extends from nucleotides 1648 to 2361 and is predicted to encode a 200-codon polypeptide that exhibits ~40% identity over the entire sequence to the N-terminal half of the gamma subunit of elongation factor 1 (25). ORF2 begins at nucleotide 3170 and extends in the same direction as *verA* through the end of the sequenced region; the 3′ end of the gene has not been sequenced yet. The predicted >380-codon ORF exhibited ~30% identity to several cytochrome P-450 monooxygenases (42).

The predicted amino acid sequence of the *A. nidulans verA* gene product and a comparison with the *A. parasiticus ver-1* gene product are shown in Fig. 2 (34). As Fig. 2 shows, the *verA* and *ver-1* gene products of *A. nidulans* and *A. parasiticus* are closely related. A search of various protein and DNA data bases revealed that the *A. nidulans* gene product exhibited homologies to products of several genes that encode NADPH-dependent reductase activities involved in polyketide biosynthetic processes. The results of previous studies had suggested that an NADPH-requiring reductase was required for conver-

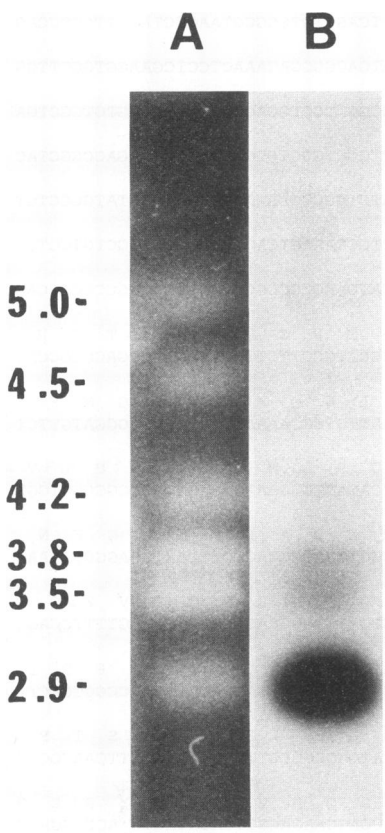

FIG. 3. *verA* is located on chromosome IV. Chromosomes of *A. nidulans* FGSC 4 (17a) were separated. The chromosomal band sizes (in megabases) are indicated on the left; chromosome IV is 2.9 Mb long. (A) Ethidium bromide-stained gel. (B) Hybridization of chromosomes to pNK10.

sion of versicolorin A to ST in the ST-AF pathway (5). The putative NADPH binding site, GXGXXA, is underlined in Fig. 2.

pNK10 (Fig. 1) was used to probe a contour-clamped homogeneous electric field gel blot of the eight separated *A. nidulans* chromosomes to determine the genomic position of *verA*. Figure 3 shows that the *verA* probe hybridized most strongly to chromosome IV, the smallest chromosome of wild-type *A. nidulans* (6). We also observed very weak hybridization to other chromosomes, most notably the chromosome doublets at 3.8 and 3.5 Mb (6), and this hybridization was thought to be nonspecific. A nearly complete overlapping cosmid map of *A. nidulans* chromosome IV has been constructed (29a), and further analysis revealed that the *verA*-containing cosmid is located within a contig that includes another cloned gene, *bimD* (13), which has been mapped to the designated left end of chromosome IV (25a).

***verA* is required for ST production.** To determine whether *verA* is required for ST production, we constructed an *A. nidulans* strain containing an insertion in the *verA* coding region. An *argB2* mutant (rM31) was transformed with pJK2 or pSalArgB, and *argB*$^+$ transformants were selected. Genomic DNAs from 51 pJK2 transformants were digested with *Hin*dIII and probed with a 1.8-kb *Pst*I fragment from pNK8 (Fig. 1). Seven transformants contained a *Hin*dIII fragment that was 3.4 kb long (the size of the *Hin*dIII fragment in pJK2) and hybridized to the probe, as predicted for a *verA* gene disruption

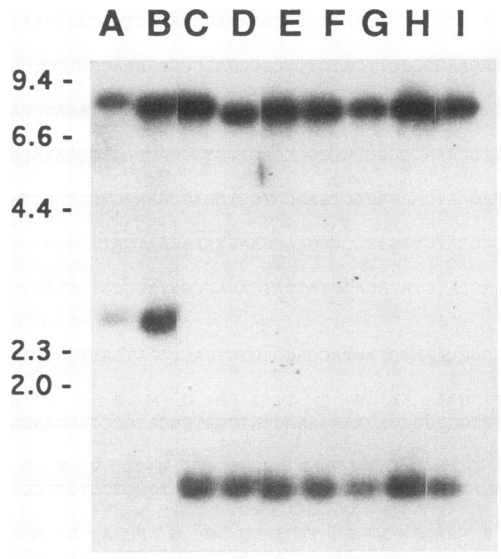

FIG. 4. Southern analysis of disrupted *verA* transformants. Genomic DNAs were digested with *Eco*RV and probed with pNK8 (Fig. 1), which contains the 3′ end of *verA*. Fragment sizes for wild-type *verA* (lanes A and B) were predicted to be 7.5 and 2.5 kb. Fragment sizes for disrupted *verA* were predicted to be 7.5 and 1.7 kb (lanes C through I). Lanes A through I contained isolates rM31, TJK1, TJK2, TJK6, TJK8, TJK10, TJK4, TJK11, and TJK7, respectively. The positions of lambda *Hin*dIII markers are indicated on the left.

event, while the remainder of the transformants contained the 6.6-kb *Hin*dIII fragment predicted for wild-type *verA* (6.6 kb was the size of the pNK10 *Hin*dIII fragment). Genomic DNAs from the seven putative *verA*-disrupted transformants were then digested with *Eco*RV and probed with pNK8. As Fig. 4 shows, all seven of these strains (TJK2, TJK4, TJK6, TJK7, TJK8, TJK10, and TJK11) contained two hybridizing fragments (1.7 and 7.5 kb), as predicted for a *verA* gene disruption. Two strains containing wild-type *verA*, rM31 and TJK1, contained the expected 2.5- and 7.5-kb fragments.

A TLC analysis of extracts from fungal strains grown under ST-inducing conditions was performed with strains FGSC 26 and TJK1 and the seven *verA* disruption strains described above. As Fig. 5 shows, none of the *verA*-disrupted transformants produced visible ST; instead, all of the strains accumulated an orange pigment that migrated to the same spot as the versicolorin A standard on TLC plates developed in benzene-glacial acetic acid (95:5, vol/vol). Only strains TJK1 and FGSC 26 produced visible ST on the TLC plates. An HPLC analysis of the same extracts revealed that there was a 200-fold to nearly 1,000-fold reduction in the ST concentration in the seven *verA* disruption strains. In this experiment the isolates were grown on 3 g of oatmeal for 1 week, and strain TJK1, which contained the wild-type *verA* gene, produced 924.0 μg of ST, while strains TJK2, TJK4, TJK6, TJK7, TJK8, TJK10, and TJK11, which contained a disrupted *verA* gene, produced 4.5, 4.0, <0.5, 2.0, 5.5, 5.0, and <0.5 μg of ST, respectively (the limit of detection was 0.5 μg of ST).

DISCUSSION

Aspergillus spp. constitute a major portion of the mycotoxin-producing fungi found worldwide. Several food- and feed-contaminating species, including *A. flavus*, *A. nidulans*, and *A. parasiticus*, are also important industrial fermentation organ-

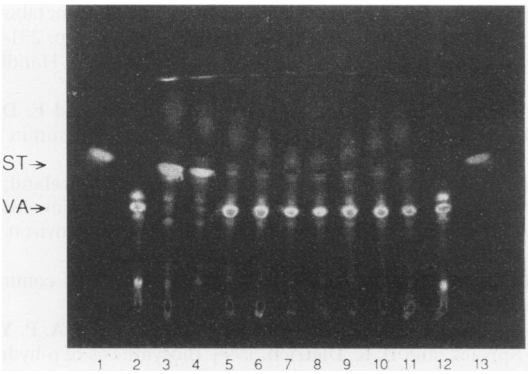

FIG. 5. *verA* disruptants do not make ST. The TLC plate was developed in benzene-glacial acetic acid (95:5, vol/vol). *A. nidulans* FGSC 26 (lane 3) and *A. nidulans* transformant TJK1 (lane 4) produced large quantities of ST, whereas transformants TJK2, TJK4, TJK6, TJK7, TJK8, TJK10, and TJK11 (lanes 5 through 11, respectively) did not produce ST but instead accumulated versicolorin A (VA). A pigment that migrated to the same spot as ST in lanes 5 through 11 was not ST as it fluoresced blue or green, whereas ST fluoresced yellow after treatment with $AlCl_3$ (38). In addition, the blue or green pigment was also produced along with ST in strains FGSC 26 and TJK1. Lanes 1 and 13 contained the ST standard and lanes 2 and 12 contained the VA standard.

isms (9, 30). An examination of the results of epidemiological studies, feeding studies, and biochemical and chemical studies has established that ST, AF, and related *Aspergillus* polyketides cause mammalian hepatocarcinomas (1, 27) and should be classified as carcinogens (16, 33). Consequently, strict national and international standards of allowable AF levels (more commonly analyzed than ST levels) in food supplies have been imposed to protect public health.

One approach to controlling AF and ST production is to understand the mechanisms that regulate production of enzymes in the pathway. Recent molecular studies have led to identification and isolation of genes and enzymes implicated in the AF biosynthetic pathway in *A. flavus* and *A. parasiticus* (10, 11, 23, 29, 34, 41, 43). However, molecular manipulations of genes in these asexual species are not easy. In fact, gene disruption techniques have only recently been described for *A. parasiticus* (40), and such techniques have not yet been described for *A. flavus*. To overcome these difficulties, we studied AF-ST regulation in *A. nidulans*, a genetically tractable relative of *A. parasiticus* and *A. flavus* for which molecular approaches are well developed (26, 39). In this study we found that *A. parasiticus ver-1*, a gene implicated in the conversion of versicolorin A to ST, has a homolog, *verA*, in *A. nidulans* and that *verA* exhibits 85% amino acid identity with *ver-1*. Disruption of *verA* in *A. nidulans* resulted in a loss of ST production and accumulation of versicolorin A, the immediate precursor of ST. This result is particularly important because, although *ver-1* was isolated from *A. parasiticus* on the basis of its ability to complement a mutation resulting in accumulation of versicolorin A, it has not been demonstrated that *ver-1* is required for AF biosynthesis by gene disruption.

A number of genes required for production of secondary metabolites in bacteria (e.g., polyketides in *Streptomyces* spp. [20]) and in fungi (18, 36) are found in clusters. Recent data support the notion that ST and AF genes are also probably clustered in *A. flavus*, *A. nidulans*, and *A. parasiticus* (10, 17a, 35). A sequence analysis of the DNA region immediately downstream of *verA* revealed two additional potential ORFs (Fig. 2). One of these ORFs is predicted to encode a polypeptide that is closely related to several cytochrome P-450 monooxygenases (42). Monooxygenase activities are proposed to be required for several steps in the ST-AF pathway (5), leading to the suggestion that this putative gene is also important for ST biosynthesis. The second ORF identified in this region exhibits a high degree of similarity to the gamma subunit of elongation factor 1 (25). What, if any, role this ORF plays in ST-AF biosynthesis is not clear. We have recently identified transcripts which accumulate during ST production from these DNA regions (21a) and will determine their function in future experiments.

Our placement of *verA* on linkage group IV has helped in directing further linkage searches of *verA* to other known genes on this chromosome. Linkage data traditionally have been obtained in *A. nidulans* through sexual crosses, but recent efforts directed toward creating an *A. nidulans* genomic contig map have allowed detailed physical placement of genes on individual chromosomes (6, 7). On the physical map *verA* is next to *bimD* on one end of chromosome IV. The location of *verA* can be confirmed by sexual crosses. The linkage information obtained from *verA* and additional ST genes in *A. nidulans* will be helpful in directing ST-AF linkage studies in *A. flavus* and *A. parasiticus*, in which gene mapping studies are limited to parasexual studies and electrophoretic karyotyping (4, 22).

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